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RTS,S/AS01$_E$ malaria vaccine induces IgA responses against CSP and vaccine-unrelated antigens in African children in the phase 3 trial

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ABSTRACT

Background: The evaluation of immune responses to RTS,S/AS01 has traditionally focused on immunoglobulin (Ig) G antibodies that are only moderately associated with protection. The role of other antibody isotypes that could also contribute to vaccine efficacy remains unclear. Here we investigated whether RTS,S/AS01$_E$ elicits antigen-specific serum IgA antibodies to the vaccine and other malaria antigens, and we explored their association with protection.

Methods: Ninety-five children (age 5–17 months old at first vaccination) from the RTS,S/AS01$_E$ phase 3 clinical trial who received 3 doses of RTS,S/AS01$_E$ or a comparator vaccine were selected for IgA quantification 1 month post primary immunization. Two sites with different malaria transmission intensities (MTI) and clinical malaria cases and controls, were included. Measurements of IgA against different constructs of the circumsporozoite protein (CSP) vaccine antigen and 16 vaccine-unrelated *Plasmodium falciparum* antigens were performed using a quantitative suspension array assay.

Abbreviations: AMA, apical membrane protein; APRIL, A proliferation-inducing ligand; BAFF, B-cell-activating factors; BS, blood stage; CelTOS, Cell-Traversal Protein for Ookinetes and Sporozoites; CHMI, controlled human malaria infection; CSP, circumsporozoite protein; EBA, erythrocyte binding antigen; EXP, exported protein; GST, Glutathione S-transferase; Ig, immunoglobulin; LS, liver stage; LSA, liver stage antigen; MSP, merozoite surface protein; MTI, malaria transmission intensity; NAI, naturally acquired immunity; PE, pre-erythrocytic; qSAT, quantitative suspension array assay; Rh, reticulocyte binding protein homologue; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SSP, sporozoite surface protein; TACI, transmembrane activator and calcium-modulating cyclophilin-ligand interactor; TGF-β1, transforming growth factor β1.

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Results: RTS,S vaccination induced a 1.2 to 2-fold increase in levels of serum/plasma IgA antibodies to all CSP constructs, which was not observed upon immunization with a comparator vaccine. The IgA response against 13 out of 16 vaccine-unrelated *P. falciparum* antigens also increased after vaccination, and levels were higher in recipients of RTS,S than in comparators. IgA levels to malaria antigens before vaccination were more elevated in the high MTI than the low MTI site. No statistically significant association of IgA with protection was found in exploratory analyses.
Conclusions: RTS,S/AS01$_E$ induces IgA responses in peripheral blood against CSP vaccine antigens and other *P. falciparum* vaccine-unrelated antigens, similar to what we previously showed for IgG responses. Collectively, data warrant further investigation of the potential contribution of vaccine-induced IgA responses to efficacy and any possible interplay, either synergistic or antagonistic, with protective IgG, as identifying mediators of protection by RTS,S/AS01$_E$ immunization is necessary for the design of improved second-generation vaccines.
Clinical trial registration: ClinicalTrials.gov: NCT008666191.

1. Introduction

The number of malaria cases globally was estimated to be 228 million in 2018, 93% in Africa [1], and most of them caused by the *Plasmodium*'s deadliest species, *Plasmodium falciparum* [2]. In endemic settings, infections become asymptomatic with age and continuous exposure to *P. falciparum* as a result of naturally acquired immunity (NAI) that is rarely sterilizing [3]. NAI is considered to be mainly mediated by IgG [4] but other isotypes such as IgM, IgE and IgA can also be induced upon natural exposure [5–7] although their relevance is less clear.

IgA is well known for being the principal antibody isotype present in the mucosal surfaces as a first line of defence [8]. However, IgA in serum is the second most abundant isotype after IgG (2–3 mg/mL) [8]. Serum IgA protects against invasive pathogens that traverse mucosal barriers and can mediate protection through the interaction with specific receptors of the immune system. Recently, IgA has gained appreciation due to the protective effects of monoclonal IgA against tumour cells, intracellular viruses as rotaviruses and bacteria such as *Mycobacterium tuberculosis* [9–11]. IgA mediates phagocytosis and killing of bacteria such as *Bordetella pertussis*, *Streptococcus pneumoniae,* and *Neisseria meningiditis* [12–14]. Furthermore, IgA is induced in the natural course of the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-Cov-2) infection and has been linked to disease severity [15]. IgA response to SARS-CoV-2 is stronger and more prolonged than that of IgM [15,16]. IgA effector functions are mediated via the IgA Fc receptor called FcαRI which is expressed on neutrophils, monocytes, macrophages and eosinophils, [17]. Its interaction with monomeric IgA induces an inhibitory signal as FcαRI is a low affinity receptor. However, when IgA forms immunocomplexes during an infection, it binds with higher avidity to the receptor resulting in the induction of pro-inflammatory responses [19]. The effector processes elicited are antibody-dependent cellular cytotoxicity (ADCC), superoxide generation, release of cytokines, and antigen presentation [19]. Indeed, Kupffer cells from the liver (specialized FcαRI + macrophages) can eliminate IgA-coated microorganisms from the bloodstream [19].

Few studies have investigated IgA responses in malaria-exposed subjects and the conclusions about their protective role are contradictory. In endemic settings in India, elevated levels of *P. falciparum*-specific IgA were detected in some individuals [6]. In another study in India, 20% of individuals had *P. falciparum*-specific IgA, which negatively correlated with IgM levels and were age-dependent [20]. In a study in Brazil, IgA predominated in children with five or less clinical infections and decreased in those with NAI and asymptomatic malaria [21]. Recently, IgA directed to the erythrocyte binding antigen (EBA) region IV ligand was found to inhibit merozoite invasion in mice [23]. To explore the mechanism(s) by which IgA may mediate a protective effect, a recombinant IgA against a single blood stage (BS) antigen (merozoite surface protein [MSP]1$_{19}$) was developed and tested in mice transgenic for FcαRI gene but no protection was observed [22]. In a phase 2b clinical trial of a candidate malaria vaccine containing the apical membrane antigen (AMA)1 and MSP1, IgA specific for these *P. falciparum* BS antigens was induced in most volunteers. Its role in protection was not assessed [24]. Even though IgA specific for MSP1 was not protective, there is no clear evidence to discard the potential role in protection of this immunoglobulin in malaria.

RTS,S/AS01$_E$ (Mosquirix™), the most advanced malaria vaccine at present [25], provided an estimated 55% protection in children aged 5–17 months over 12-months of follow up post primary immunization in a multicentre phase 3 trial in Africa [25–29]. RTS,S is a pre-erythrocytic (PE) vaccine that includes 19 NANP repeats of the central region of the circumsporozoite protein (CSP) and its C-terminal region (C-term), fused to the hepatitis B surface antigen [30], and formulated with the AS01$_E$ adjuvant [31]. RTS,S/AS01$_E$ vaccination induces potent IgG responses that are associated with efficacy but an absolute correlate of protection has not been established [26,32–34]. We previously showed that RTS,S/AS01$_E$ immunization affected IgG responses to non-vaccine *P. falciparum* PE and BS antigens, and that this could have an impact on protective immunity to malaria [35]. Little is known about IgA responses to RTS,S/AS01$_E$, although CSP-specific IgA secreting cells have been reported in vaccinated malaria-naïve adults [36]. In controlled human malaria infection (CHMI) studies with naïve adults, IgA2 levels specific for CSP correlated with protection and this was also tied to higher concentrations of IgA1 to CSP [37]. Given a possible role for serum IgA in malaria protection, we investigated whether IgA responses are induced against CSP in RTS,S-vaccinated children, which could potentially contribute to efficacy. We further investigated whether the interaction with NAI responses previously shown for IgG was also observed with IgA.

In this study, we set out to quantify the levels of total IgA in serum/plasma samples from RTS,S-vaccinated and non-vaccinated (comparator) children from the multicentre paediatric African phase 3 trial of RTS,S/AS01$_E$ [25–29] before and after primary immunization. We included children from two African sites with different MTI, and who were included in our previous immunology studies [32,38]. We explored association of IgA levels with site, sex and malaria exposure and protection. To this end, we developed and optimized a quantitative suspension array assay (qSAT) to measure antigen-specific IgA against multiple *P. falciparum* antigens.

2. Materials and methods

2.1. Study design and subjects

We analysed IgA responses in 95 children (age 5–17 months) randomly selected from a subset of volunteers participating in a prior set of analyses [33,35,38] within the immunology ancillary study (MAL067) to the phase 3 trial (MAL055). The reason to include this subset of children with previous data was to gather information about immune features of the same individuals to conform a comprehensive picture of the RTS,S/AS01E elicited immunity. However, we only included a subset of these due to the exploratory nature of the study and because the sample size was adequate to answer our primary objective and to establish a basis for further larger studies. Samples were collected at month (M)0 (before primary vaccination) and at M3 (one month after the 3rd dose) in two sites: Manhiça (plasma) in Mozambique, a low MTI area, and Kintampo (serum) in Ghana, a high-moderate MTI area [25,29,30]. These two sites were prioritized due to higher availability of sufficient numbers and volumes of samples from both study visits and age cohorts. Clinical malaria was determined by passive case detection (PCD) starting 14 days after sample collection at M3 for the subsequent 12 months. Among the 95 children included in the study, 66 were vaccinated with RTS,S/AS01$_E$ (40 from Manhiça and 26 from Kintampo) and 29 received a comparator vaccine (15 from Manhiça and 14 from Kintampo). Among the RTS,S/AS01$_E$ vaccinated, 29 children had malaria (10 from Manhiça and 19 from Kintampo) whereas among the comparator group, 18 children were malaria cases (5 from Manhiça and 13 from Kintampo) (Fig. 1). The study protocol was approved by the Ethics Committees from Spain, Mozambique and Ghana, and written informed consent was obtained from parents or guardians before starting study procedures.

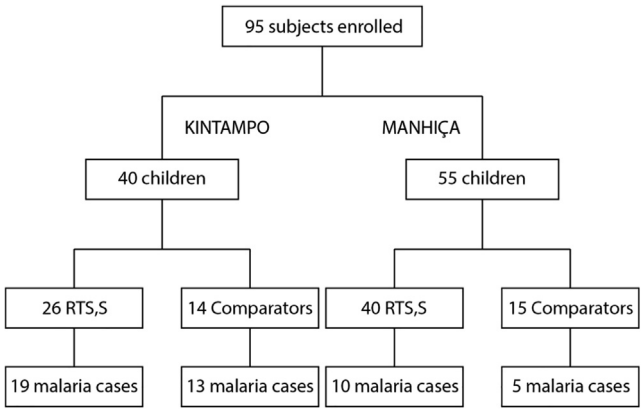

Fig. 1. Flowchart of the study design.

2.2. Antigens

Nineteen *P. falciparum* antigens were selected for the multiplex qSAT panel (Table 1) based on previous data on IgG responses in RTS,S/AS01$_E$ vaccinees from our group, and on IgA data from the literature. The panel included 6 antigens from the PE stage and 12 from the BS. α-Gal (Galα1-3 Galβ1-4GlcNAc-BSA) was added to the panel because antibodies against this carbohydrate antigen have recently been associated with malaria protection [39,40].

2.3. Antibody assays

Antigens were coupled to MAGPLEX 6.5 μm COOH-microspheres from Luminex Corporation (Austin, TX) as explained elsewhere [55]. Antigen-coupled beads were added to a 96-well μClear® flat bottom plate (Greiner Bio-One, Frickenhausen, Germany) at 1000 beads/analyte/well in a volume of 40 μL/well of phosphate buffered solution with 0.05% Tween 20 (PBS-BN). For more accurate IgA measurements, all samples were diluted in GullSORB™ IgG Inactivation Reagent (Meridian Bioscience™) prior to testing for IgA levels, in order to deplete IgG and reduce competition for the same epitopes that might interfere with the quantification of antigen-specific IgA [56]. Thus, 40 μL/well of the diluted sample (final dilutions: 1/150 and 1/12150) in PBS-BN with GullSORB™ at a 1:10 concentration were added to the plates and incubated at 4 °C overnight (ON) in a shaker at 600 rpm and protected from light. After the ON incubation, beads were washed three times with 200 μL/well of PBS-BN using a manual magnetic washer platform (Bio-Rad, Hercules, CA, USA). A hundred microliters of a goat anti-human IgA (α-chain specific) F(ab′)2 fragment-biotinylated secondary antibody (Sigma, SAB3701227) was added (1/250 in PBS-BN) to all wells. Plates were incubated at RT for 45 min at 600 rpm and protected from light and then washed three times. A 100 μL of streptavidin-R-phycoerythrin (Sigma, 42250) were added (1/1000 in PBS-BN) to all wells and incubated at RT for 30 min at 600 rpm protected from light. Plates were washed three times and beads were resuspended in 100 μL of PBS-BN and read using a Luminex xMAP™100/200 analyser (Luminex Corp., Texas). At least 50 beads per analyte were acquired per sample. Crude median fluorescent intensity (MFI) and background fluorescence from blank wells were exported using the xPONENT software.

The detection range of the assay, adequate sample dilutions and assay reproducibility, were established before analysing test samples. A pool made of serum samples from CSP IgG high responders from the same study was prepared to construct a standard curve with 12-serial dilutions starting at 1/150 in a 1:3 sample:buffer passage. Plasma from malaria unexposed adults (1/150) were used as negative controls. Two blank control wells with beads in PBS-BN were set up to measure background signal.

Samples were tested in two separate experiments comparing IgA levels at M0 and M3. The first experiment included 66 RTS,S vaccinees and was designed to evaluate whether RTS,S induced antigen-specific IgA responses at M3. The second experiment included 28 RTS,S-vaccinees from the first experiment and 29 comparators and intended to assess whether the IgA responses observed in the first experiment were RTS,S-specific and not just due to malaria exposure.

2.4. Data pre-processing

MFI data was normalised across plates using the standard curves per each antigen. For each plate, a normalization factor was calculated as the average of the MFIs corresponding to the maximum slope of the curves from all plates divided by the MFI corresponding to the maximum slope of the curve from each plate. MFIs of each plate were multiplied by the resulting normalisation factor. Two different dilutions were used in the study and one of them was chosen to further perform the analysis. The chosen dilution used for the analysis was antigen and plate specific and was calculated using the standard curves. The dilution with an MFI closer to the MFI corresponding to the maximum slope was selected. To obtain the final MFI values taking into account the dilution selected, a correction factor was used based on the maximum slope of the curve for each antigen and plate. The formula used to obtain the final corrected MFI is $y = mx + n$, where y is the final corrected MFI value, m is the maximum slope, x is the $\log_{10}$ dilution factor, and n is the normalised MFI corresponding to the selected dilution

Table 1
Antigens included in the multiplex panel.

Antigens	TAG	Life cycle stage	Comments	References
Pre-erythrocytic				
CelTOS		Sporozoite	Sporozoite exposure; Cell-Traversal Protein for Ookinetes and Sporozoites	[41]
CSP full length		Sporozoite	Component of RTS,S	[42]
CSP NANP repeat	GST-Fused	Sporozoite	Component of RTS,S	[32]
CSP C-term	GST-Fused	Sporozoite	Component of RTS,S	[32]
SSP2		Sporozoite	Sporozoite exposure; Sporozoite surface protein 2	[43]
LSA1		Liver	Infected hepatocytes; liver surface antigen 1	[44]
Blood stage				
AMA1		Merozoite	Involved in erythrocyte invasion; apical membrane antigen 1	[45]
EBA140	GST-Fused	Merozoite	Involved in erythrocyte invasion; erythrocyte binding antigen 140	[46]
EBA175 R3-5	GST-Fused	Merozoite	Involved in erythrocyte invasion	[46]
EXP1		Merozoite	Involved in erythrocyte invasion, exported protein 1	[47]
MSP1 Block 2	GST-Fused	Merozoite	Involved in erythrocyte invasion; merozoite surface protein 1	[48]
$MSP1_{42}$		Merozoite	Involved in erythrocyte invasion	[48]
MSP3 3C		Merozoite	BS exposure	[49]
MSP5		Merozoite	BS exposure	[50]
MSP6	GST-Fused	Merozoite	BS exposure	[51]
Rh2 (2030)	GST-Fused	Merozoite	Involved in erythrocyte invasion; reticulocyte binding protein homologue 2	[52]
Rh4.2	GST-Fused	Merozoite	Involved in erythrocyte invasion	[53]
Rh5		Merozoite	Involved in erythrocyte invasion	[54]
Other antigens				
α-Gal			Involved in malaria protection	[39,40]

factor. Final normalised and corrected MFI data was log_{10}-transformed to perform the statistical analysis.

2.5. Statistical analysis

IgA levels (log_{10} MFI) were compared between vaccine groups by t-tests or between timepoints by paired t-tests, and represented by boxplots depicting means, medians and interquartile ranges. Comparisons between timepoints were also stratified by vaccine group, site and sex. P-values were corrected for multiple comparisons by the Benjamini-Hochberg approach and considered significant after adjustment when < 0.05. Fold-changes between timepoints were calculated as the difference between log_{10} MFI levels at M3 vs log_{10} MFI levels at M0. Correlations between IgA and IgG levels (log_{10} MFI) were assessed using scatterplots and the Spearman method and raw p-values < 0.05 were considered significant due to the exploratory nature of the analysis. Data analysis was performed in R software (version 3.6.1.) [57], and the data were managed using devtools package (version 2.2.2.) [58].

3. Results

3.1. Increased IgA levels to CSP after RTS,S vaccination

Three doses of RTS,S/$AS01_E$ administered at one-month intervals induced a statistically significant increase of IgA levels (log_{10}-MFI) against CSP one month after the last dose. IgA levels to CSP full length (FL), NANP repeat and C-term constructs, increased from baseline (M0) to post-vaccination (M3) ($p < 0.001$; Fig. 2). The highest increase (2-fold) was recorded for CSP FL followed by NANP repeat (1.5-fold) and then C-term (1.2-fold), whose levels overlapped between baseline and post-vaccination. RTS,S vaccinees had higher IgA levels than comparator vaccinees for all CSP constructs at M3 ($p < 0.001$). Children who received the comparator vaccine showed no increase after vaccination (Fig. 2B).

3.2. Increased IgA levels to vaccine-unrelated antigens following RTS,S vaccination

RTS,S vaccination in children significantly increased IgA levels to most vaccine-unrelated antigens from pre- to post-vaccination, including α-Gal, AMA1, CelTOS, EBA140, EBA175, LSA1, MSP1 block 2, MSP3 3C, MSP5, MSP6, Rh2, Rh4.2, Rh5 and SSP2/TRAP ($p < 0.001$; Fig. 3). IgA response did not increase for EXP1 and $MSP1_{42}$ antigens.

Further evaluation of IgA responses was performed with a smaller set of RTS,S vaccinees and including children from the comparator vaccine group. IgA levels against α-Gal, CelTOS, EBA140, LSA1, MSP1 block 2, MSP6, Rh4.2, Rh5 ($p < 0.002$; Fig. 4) increased after RTS,S vaccination. In the case of α-Gal and MSP6, IgA levels were not statistically significant different between comparator and RTS,S vaccinated children at M3. No differences were observed for AMA1, EBA175, EXP1, $MSP1_{42}$, MSP5, Rh2 and SSP2/TRAP IgA responses.

3.3. IgA levels by sites of different MTI, sex and malaria disease

IgA responses after RTS,S/$AS01_E$ vaccination to the CSP constructs and to most of the *P. falciparum* antigens were similar between sites (Fig. 5). However, Kintampo (higher MTI) tended to have higher IgA responses than Manhiça (lower MTI), although differences did not always reach statistical significance. Baseline IgA levels to AMA1, EXP1, $MSP1_{42}$ were significantly higher in Kintampo than in Manhiça ($p = 0.006$) and a similar tendency was found for LSA1 ($p = 0.056$) (Fig. 5). We did not detect any differences in IgA levels to CSP or other *P. falciparum* antigens by sex (Supplementary Fig. 1). In exploratory analysis, we did not find significant differences in antigen-specific IgA levels between malaria cases and controls (Supplementary Fig. 2).

3.4. IgA levels correlated with IgG, IgG1 and IgG3 levels against CSP antigens

Correlation of IgA with IgG [35] and IgG_{1-4} [38] levels for each of the antigens was assessed post-vaccination in 57 samples for which prior IgG data were available. IgA to CSP moderately correlated with IgG specific for CSP. IgA to NANP had moderate significant correlations with IgG to C-term and low significant correlation with IgG to CSP (Fig. 6A). IgA to NANP correlated modestly and non-significantly with IgG specific for NANP (Fig. 6A). IgA to NANP showed moderate significant correlations with IgG1 and IgG3 specific for CSP (Fig. 6B and C, respectively).

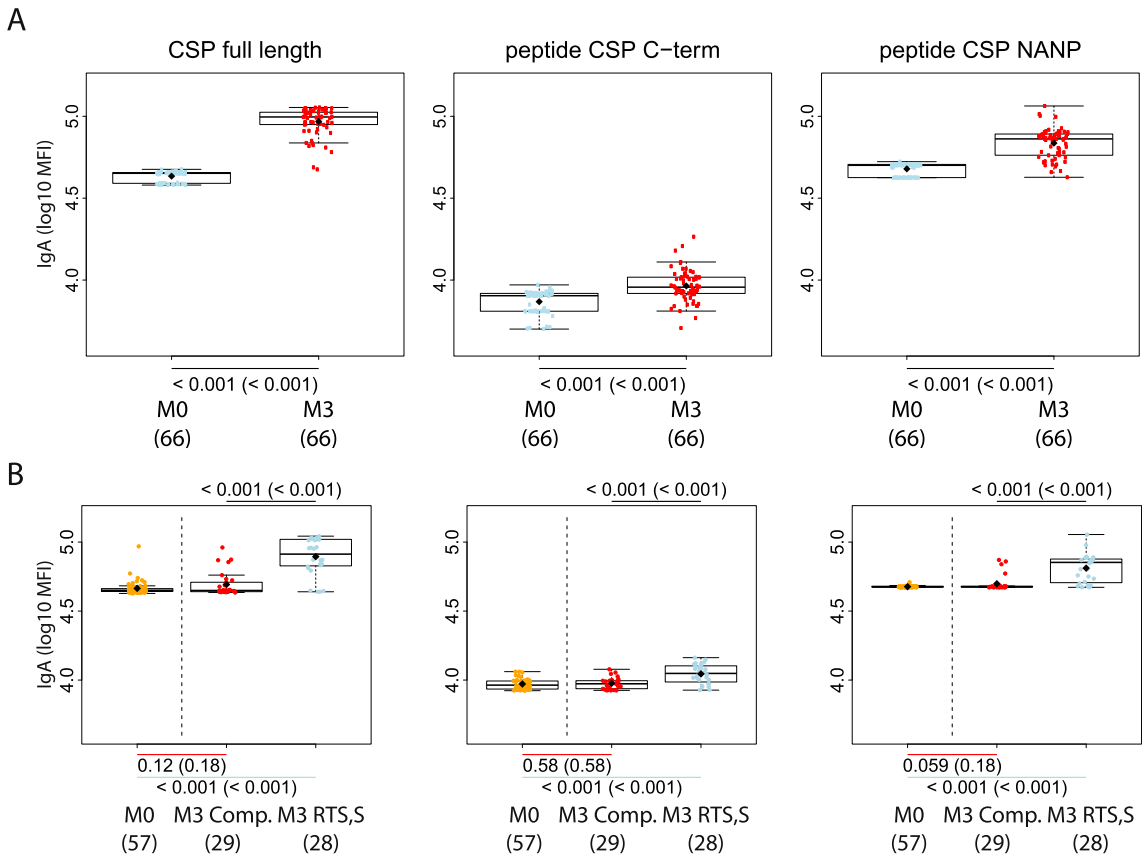

Fig. 2. RTS,S-induced IgA response to CSP antigens. Boxplots showing A) an increase in the IgA levels (median fluorescence intensity, MFI) against CSP antigens between the two timepoints month 0 (M0) and month 3 (M3, adjusted p-value < 0.001) and B) comparing RTS,S/AS01E and comparator vaccinated children at M3, showing that the increase between timepoints was only observed in the RTS,S group (p-value < 0.001). Boxes depict median MFIs, interquartile ranges (IQR) and log10 geometric mean (diamonds); the lower and upper hinges correspond to the first and third quartiles; whiskers extend from the hinge to the highest or lowest value within 1.5 × IQR of the respective hinge. Paired *t*-test (A) and *t*-test between M3 vaccinated and comparator children (B) were used to assess statistically significant differences in antibody levels between groups. *T*-test results are given as p-values and adjusted p-values for multiple testing (shown in parenthesis). Horizontal lines indicate groups compared in the *t*-test. Only adjusted p-values < 0.05 were considered. The y axis MFI is shown in log10 scale. Numbers in parenthesis indicate total of individuals in each category.

4. Discussion

Our data show that the RTS,S/AS01$_E$ vaccine elicits a robust IgA response against CSP. Very little is known about IgA responses to *P. falciparum* and its induction by malaria vaccines. To our knowledge, this is the first study showing that IgA responses can be elicited following RTS,S vaccination in a clinical trial in malaria endemic areas. The magnitude of the IgA response against CSP was particularly high for the CSP FL construct, and lower for the NANP and C-term regions. This contrasts with the IgG response that is generally higher to the immunodominant region of the molecule, the central NANP repeats. Overall, there was a poor correlation between IgA and IgG to CSP constructs, suggesting that IgA responses may be differentially regulated to IgG and its subclass responses in the vaccinated children.

Interestingly, IgA levels increased after RTS,S vaccination also for other *P. falciparum* antigens not included in the vaccine, and this appeared to be specific to RTS,S vaccination as it was not observed in comparator vaccinees. These antigens included CelTOS, EBA140, LSA1, MSP1 block 2, MSP3 3C, MSP6, Rh4.2 and Rh5, and also α-Gal, which is a widely distributed carbohydrate not specific to *P. falciparum* but thought to be present in sporozoites [39]. Some additional antigens showed increased levels when testing more RTS,S vaccinees, probably because of the increased sample size (e.g. SSP2). All of these antigens showed significant differences between RTS,S and comparator vaccinees, with the exception of α-Gal, whose increase in IgG levels has also been associated with age in children 5–17 months of age [39]. We had previously observed an increase of IgG, IgG1 and IgG3 levels to certain non-vaccine *P. falciparum* antigens after RTS,S vaccination [35,38], therefore the IgA increase was not unexpected.

On one hand, we hypothesize that RTS,S could be making the sporozoite more visible to the immune system by means of opsonisation, which could be enhancing antigen presentation and the production of IgA (and IgG) against PE *P. falciparum* antigens due to natural exposure. The RTS,S-induced antibodies against the CSP antigens could slow down the sporozoite invasion of the hepatocytes, allowing a prolonged sporozoite exposure to the immune system, thus facilitating the response to PE antigens [38]. On the other hand, the increase in IgA to *P. falciparum* BS antigens might be related to the fact that RTS,S confers partial protection and is non sterilizing, acting as a "leaky" PE vaccine. As such, it may reduce hepatocyte invasion, liver stage development and merozoite release from the liver, resulting in a low BS parasitaemia [38,59]. It is possible that high parasite densities may lead to less effective adaptive immune responses, while sustained partially controlled infection would result in low parasitaemia that could elicit enhanced IgA (and IgG) production to certain antigens. Thus, natural exposure to *P. falciparum* between M0 and M3 could induce the increase in IgA specific for BS antigens in RTS,S vaccinees [38,59]. Alternatively, the adjuvant could stimulate antigen-specific responses to natural exposure during vaccination, increasing the antibody levels [38]. However, we cannot ignore the possibility that CSP antibodies might be cross-reactive and also

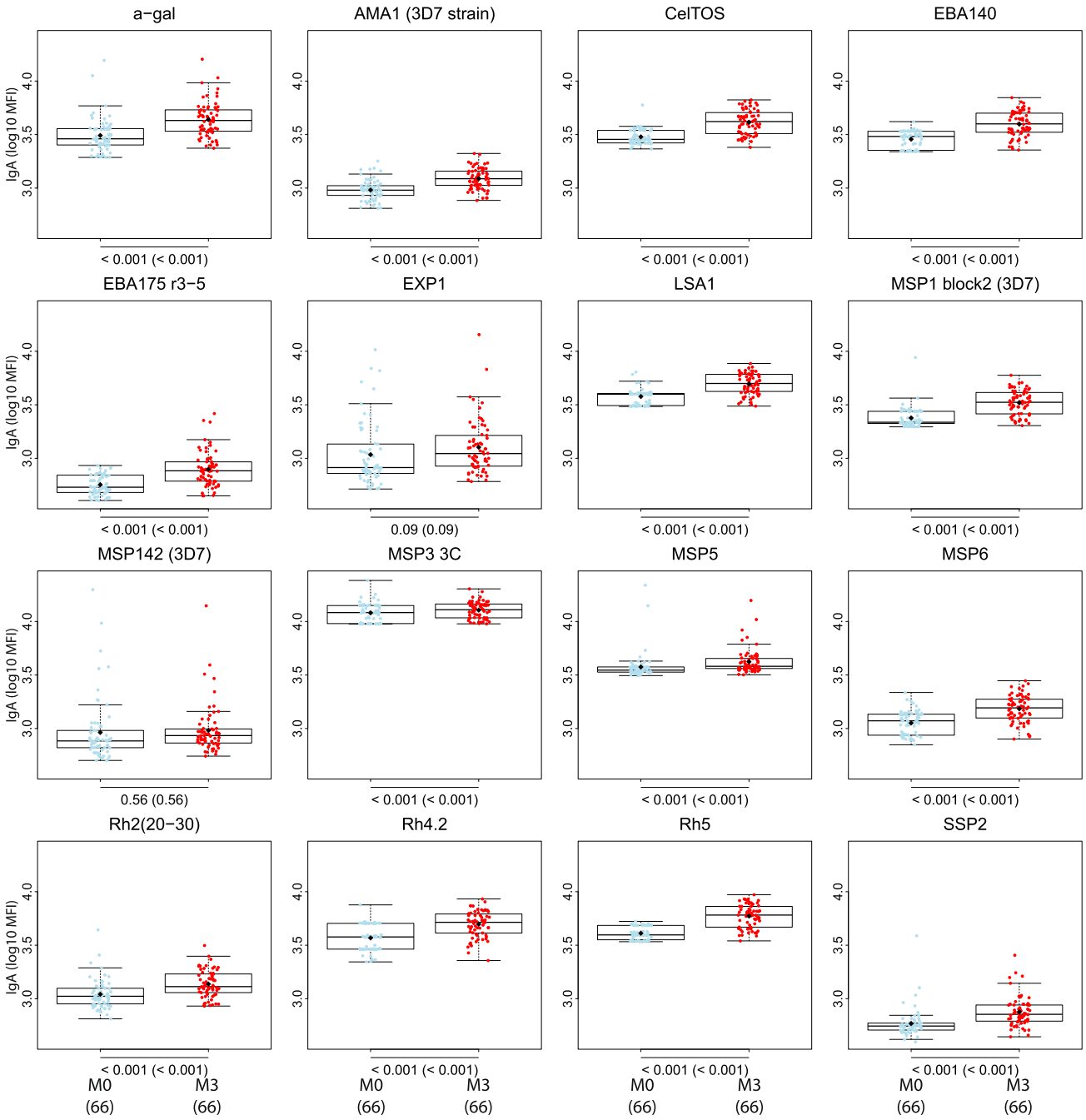

Fig. 3. IgA levels against vaccine-unrelated *P. falciparum* antigens in RTS,S/AS01$_E$-vaccinated children. IgA levels (median fluorescence intensity, MFI) were higher at month 3 (M3) compared to month 0 (M0) for α-Gal, AMA1, CelTOS, EBA140, EBA175, LSA1, MSP1 block 2, *MSP3 3C*, MSP5, MSP6, Rh2, Rh4.2, Rh5 and SSP2/TRAP (adjusted p-value < 0.001). Boxes depict median MFIs, interquartile ranges (IQR) and $\log_{10}$ geometric mean (diamonds); the lower and upper hinges correspond to the first and third quartiles; whiskers extend from the hinge to the highest or lowest value within 1.5 × IQR of the respective hinge. Paired t-tests were used to assess statistically significant differences in antibody levels between groups. *T*-test results are given as p-values and adjusted p-values for multiple testing (shown in parenthesis). Horizontal lines indicate groups compared in the *t*-test. Only adjusted p-values < 0.05 were considered. The y axis MFI is shown in $\log_{10}$ scale. Numbers in parenthesis indicate total of individuals in each category.

recognise vaccine unrelated antigens, even though there is no homology at the primary amino acid sequence level between these proteins [35]. There is some support for this idea in previous findings that asparagine rich sequences in CSP, such as NANP, give rise to cross-reactive antibodies that recognize asparagine rich sequences in BS proteins [60].

IgA has been predominantly associated only with mucosal protection and thus neglected in studies of blood-borne pathogens, but it may have a potentially protective role against *P. falciparum* infection. The biological and immune mechanisms by which B cells produce IgA to this vaccine or to *P. falciparum* infections are not known. In humans, there are two subclasses of IgA, IgA1 (monomeric) and IgA2 (dimeric) [8], but serum IgA is predominantly IgA1 (~90%). Here, we measured total IgA, but probably most of the response was IgA1, although this should be confirmed in subclass-specific assays, as function differs by subtype.

Isotype switching is initiated in activated B cells and can be induced by two pathways: T cell-dependent or T cell-

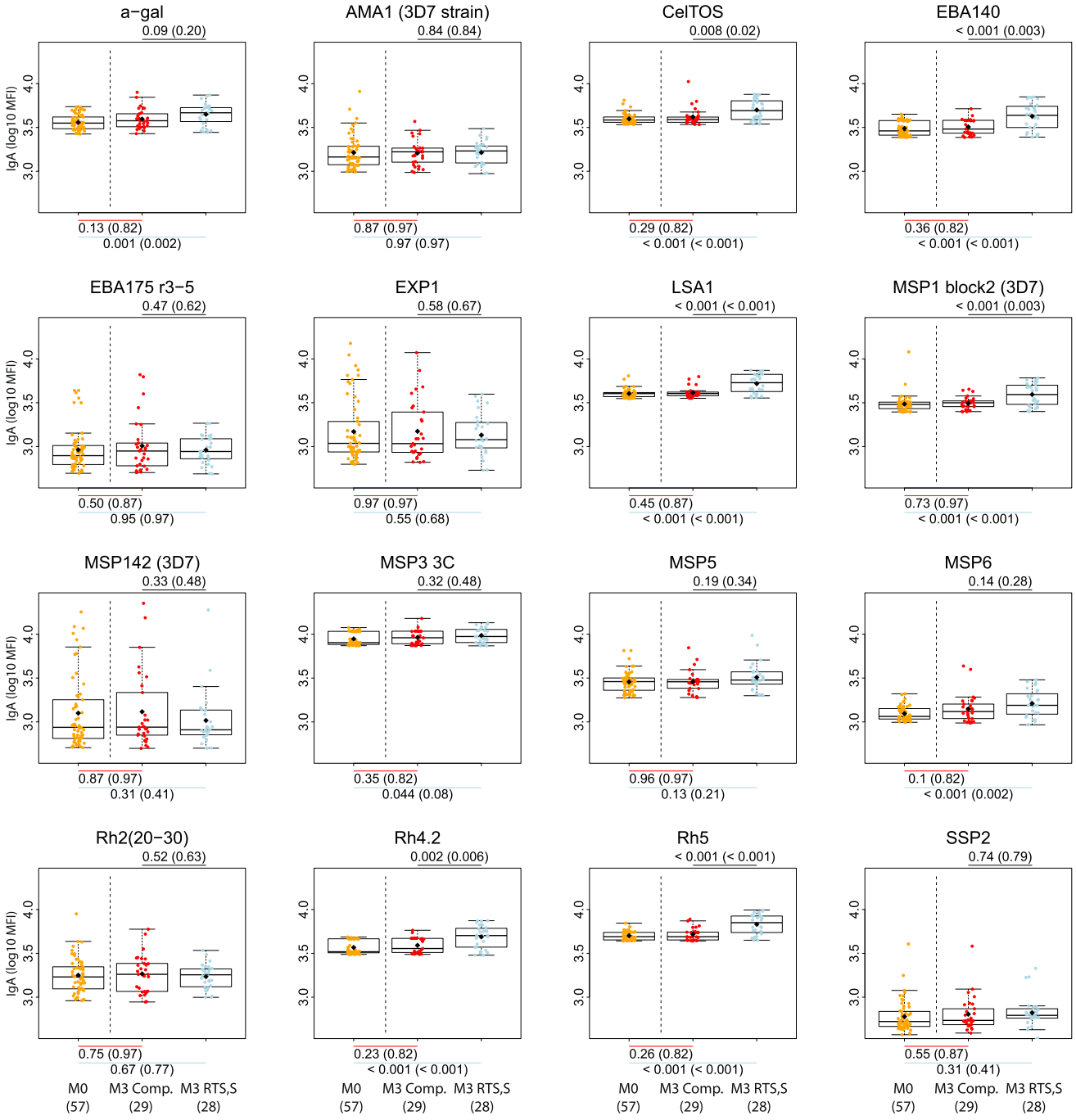

Fig. 4. IgA levels against vaccine-unrelated *P. falciparum* antigens in RTS,S/AS01$_E$- and comparator-vaccinated children. IgA levels (median fluorescence intensity, MFI) increased for some *P. falciparum* vaccine-unrelated antigens between month 0 (M0) and month 3 (M3) in RTS,S vaccinated children unlike the comparator group. These antigens were α-Gal, CelTOS, EBA140, LSA1, MSP1 block 2, MSP6, Rh4.2, Rh5 (adjusted p-value < 0.001). Boxes depict median MFIs, interquartile ranges (IQR) and $\log_{10}$ geometric mean (diamonds); the lower and upper hinges correspond to the first and third quartiles; whiskers extend from the hinge to the highest or lowest value within 1.5 × IQR of the respective hinge. *T*-test between M3 vaccinated and comparators and paired t-tests between M0 and M3 groups were used to assess statistically significant differences in antibody levels between groups. *T*-test results are given as p-values and adjusted p-values for multiple testing (shown in parenthesis). Horizontal lines indicate groups compared in the *t*-test. Only adjusted p-values < 0.05 were considered. The y axis MFI is shown in $\log_{10}$ scale. Numbers in parenthesis indicate total of individuals in each category.

independent class switching [61]. T cell-dependent IgA class switch essentially depends on two signals in addition to MHC-TCR: CD40-CD40L interaction and transforming growth factor β1 (TGF-β1) [61]. TGF-β1 is a potent immunoregulator that may downregulate IgG production and induce regulatory T cells in humans [62]. This cytokine is anti-inflammatory at high concentrations and proinflammatory at low concentrations [63] and IgA is induced at low TGF-β1 concentrations [63]. During early *P. falciparum* infection, TGF-β1 promotes T_H-1 (e.g. IFN-γ) inflammatory responses that control parasite growth. Later, TGF-β1 downregulates this response to limit pathology related to an exacerbated inflammation [64]. It might be possible that at the beginning of a malaria infection, TGF-β1 is present at very low concentrations and stimulates antibody production of IgA and IgG in a proinflammatory milieu. An inverse correlation between IgG to CSP and TGF-β1 levels has been observed after

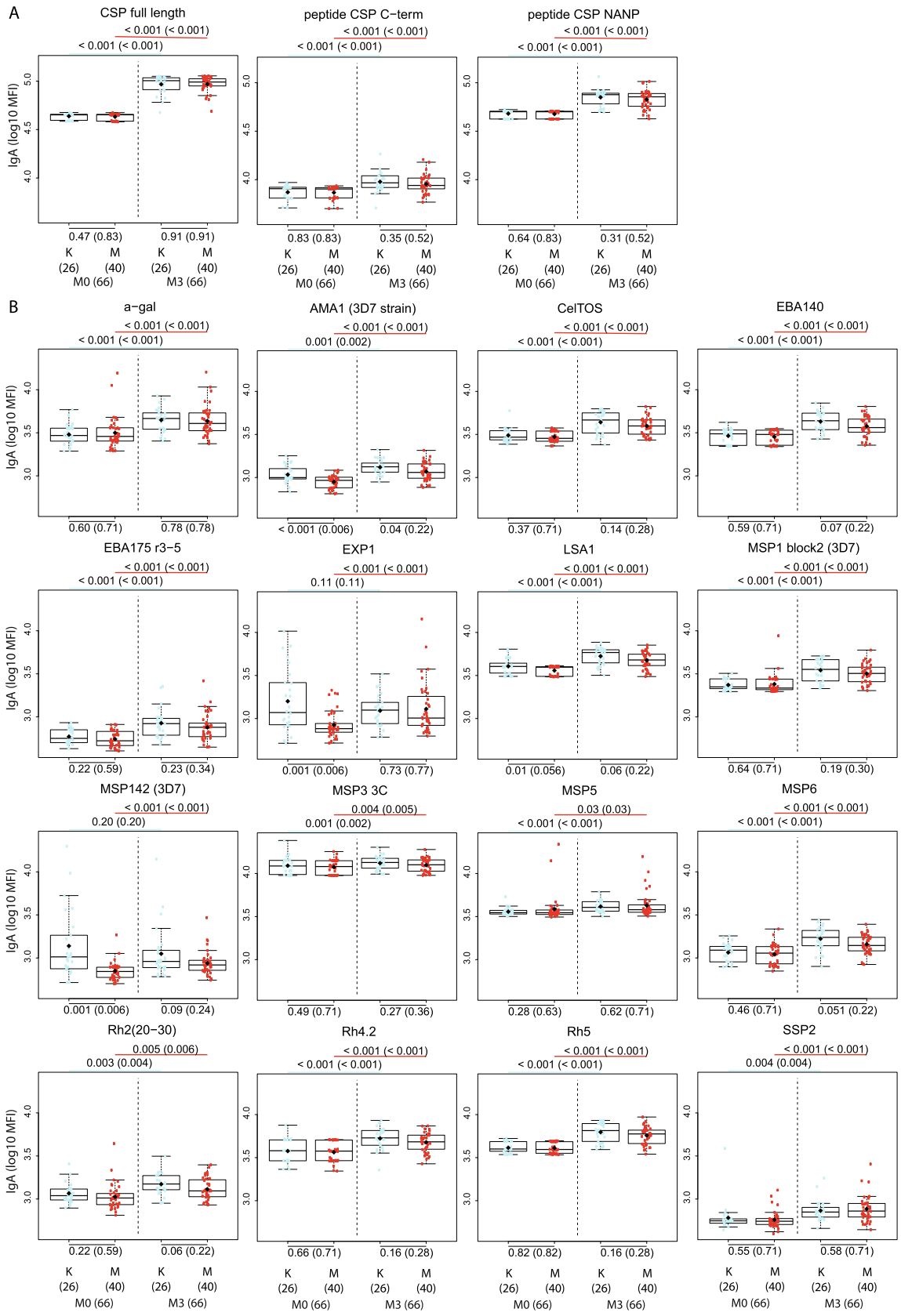

Fig. 5. Antigen-specific IgA levels stratified by site at timepoints month 0 and month 3. IgA levels (median fluorescence intensity, MFI) from Kintampo (K) and Manhiça (M) RTS,S vaccinated children are shown in the graph for CSP antigens (**A**) and vaccine-unrelated *P. falciparum* antigens (**B**). Specific IgA against AMA1, EXP1 and $MSP1_{42}$ showed higher levels in Kintampo than in Manhiça (adjusted p-value < 0.05) at M0. Boxes depict median MFIs, interquartile ranges (IQR) and $\log_{10}$ geometric mean (diamonds); the lower and upper hinges correspond to the first and third quartiles; whiskers extend from the hinge to the highest or lowest value within 1.5 × IQR of the respective hinge. Paired t-tests were used to assess statistically significant differences in antibody levels between groups. *T*-test results are given as p-values and adjusted p-values for multiple testing (shown in parenthesis). Horizontal lines indicate groups compared in the *t*-test. Only adjusted p-values < 0.05 were considered. The y axis MFI is shown in $\log_{10}$ scale. Numbers in parenthesis indicate total of individuals in each category.

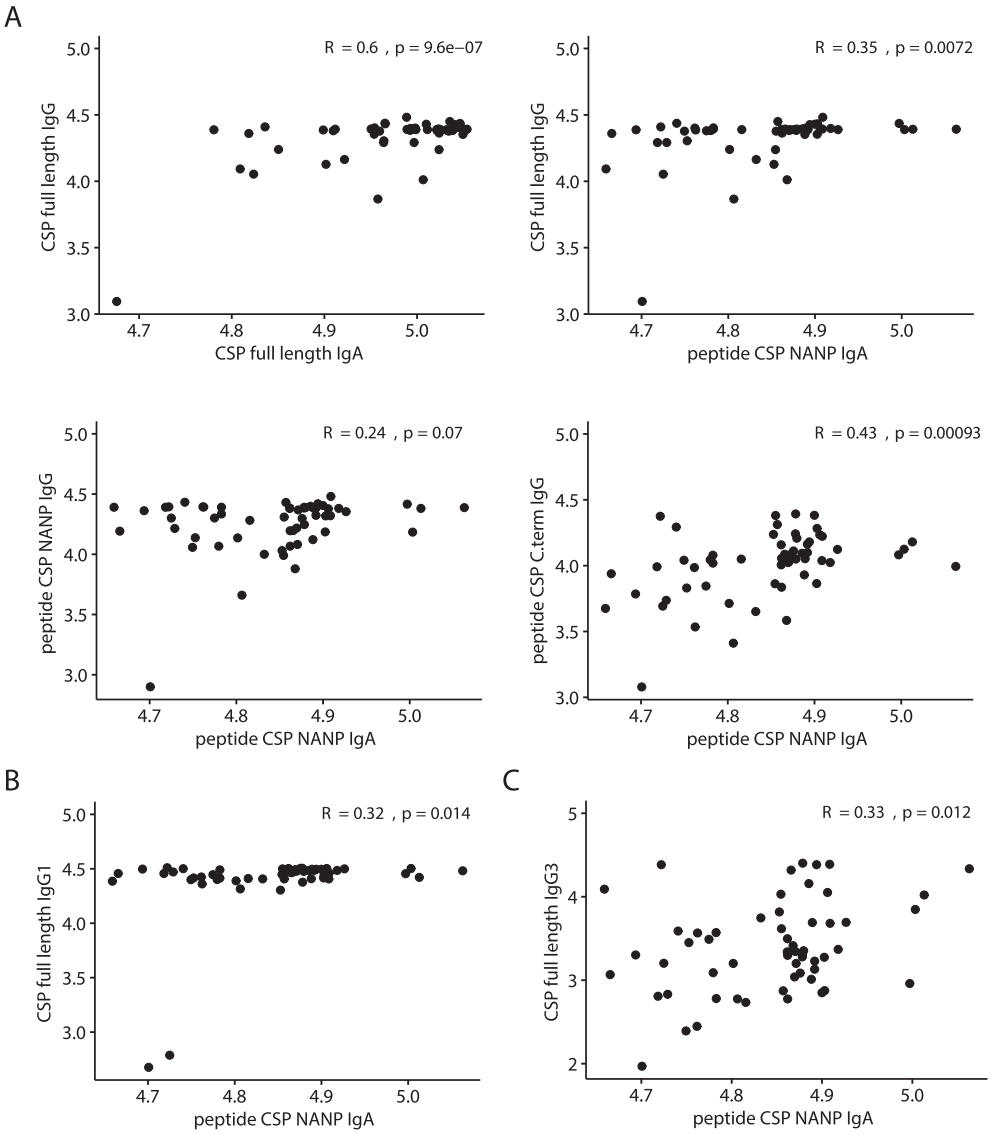

Fig. 6. Correlation scatterplots between IgA and IgG levels at month 3 in RTS,S vaccines. IgA against CSP antigens moderately or lowly correlated with IgG (**A**) IgG1 (**B**) and IgG3 (**C**) specific for the same antigens. IgA specific for CSP correlated with IgG against the same antigen. IgA specific for NANP correlated with IgG against CSP full length, NANP and C-term peptides, and with IgG3 specific for CSP full length. Both y and × axes are median fluorescent intensities (MFI) in $\log_{10}$ scale. Correlation estimate and its p-value were calculated with the Spearman method. Correlation estimate is given as (R) and p-value as (p). Low correlation: 0.2 < R < 0.39. Moderate correlation: 0.4 < R < 0.59.

RTS,S vaccination [62]. TGF-β1 levels might be low when IgG is being produced. Given the fact that IgA is induced at low concentrations of TGF-β1, IgA could also be produced in vaccinated children. T cell-dependent responses take from 5 to 7 days to develop. There are specific B cell subsets specialized in producing IgM that also class switch to IgG and IgA in a CD40L-independent manner (T-cell independent responses) [61]. This response is elicited by antigens on the surface of pathogens that are organised and repetitive and cross-link the B cell receptor, resulting in short-lived responses [65]. Some BS antigens from *P. falciparum*, such as hemozoin and the schizont fraction of *P. falciparum* antigen, are thought to induce this kind of response [66]. The highly repetitive sequence of NANP included in the RTS,S vaccine could also be inducing a T cell-independent response, which might be related to the production of short-lived antibodies and vaccine efficacy [38].

To our knowledge, serum IgA has not been studied in other parasites that infect erythrocytes. In contrast, it has been quantified in response to pathogens that infect the mucosa like *Toxoplasma gondii*, and in vaccines against this parasite [67–69]. A monoclonal IgA administered intraperitonealy protected against *Acanthamoeba keratitis* [70]. Natural infection by *Entamoeba histolytica* induced shared specificities in serum IgG and IgA in baboons [71]. Serum IgA against the amoeba *Naegleria fowleri* were higher in infected patients [72]. Importantly, IgA could also have a negative effect on protection. IgA antibodies binding to Env protein in HIV were positively correlated with risk of infection and negatively correlated with vaccine efficacy [73]. Unfortunately, our study was not designed nor powered to assess the correlation of IgA with RTS,S-induced malaria protection but we cannot discard that differences would be observed between malaria protected and non-protected in larger studies. Emerging data suggest that IgG interactions with complement and Fcγ-receptors on immune cells may be mechanisms mediating protection with RTS,S [74,75]**.** Evaluating interactions of IgA with complement and Fcα-receptors in future studies may help understand the potential role of IgA in vaccine-induced immunity.

Having observed the increase in IgA levels after RTS,S vaccination, and considering the IgA immune mechanisms described in previous studies, we postulate that IgA blood responses could have

an important role in the defence against blood borne pathogens such as *P. falciparum* and deserve further investigation.

5. Conclusion

RTS,S/AS01$_E$ elicited IgA responses against CSP constructs and vaccine-unrelated *P. falciparum* antigens, as previously observed for IgG responses, which may be related to its partial protection and 'leaky' effect, suggesting a beneficial interaction with NAI. Further studies are required to establish the dynamics of the response, including the booster dose, and its relation to vaccine efficacy. Overall, our study underscores the need to include IgA assessment and understanding in malaria vaccine and NAI studies, considering that its role in immunity may go beyond mucosal protection.

CRediT authorship contribution statement

Roger Suau: Writing - review & editing, Data curation, Formal analysis. **Marta Vidal:** Writing - review & editing, Methodology. **Ruth Aguilar:** Writing - review & editing, Methodology. **Gemma Ruiz-Olalla:** Data curation. **Miquel Vázquez-Santiago:** Writing - review & editing, Data curation, Methodology. **Chenjerai Jairoce:** Resources. **Augusto J. Nhabomba:** Resources. **Ben Gyan:** Resources. **David Dosoo:** Resources. **Kwaku Poku Asante:** Resources. **Seth Owusu-Agyei:** Resources. **Joseph J. Campo:** Resources. **Luis Izquierdo:** Resources. **David Cavanagh:** Resources. **Ross L. Coppel:** Resources. **Virander Chauhan:** Resources. **Evelina Angov:** Resources. **Sheetij Dutta:** Resources. **Deepak Gaur:** Resources. **James G. Beeson:** Resources. **Gemma Moncunill:** Writing - review & editing, Project administration. **Carlota Dobaño:** Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

The study protocol was approved by the Ethics Committees from Spain, Mozambique, and Ghana, and written informed consent was obtained from parents or guardians before starting study procedures.

Consent for publication

Not applicable.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.12.038.

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